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(54) Title: IDENTIFICATION OF TARGET BACTERIA BY FLUORESCENCE DETECTION OF PRIMER DIRECTED AMPLIFICATION PRODUCTS

(57) Abstract

The invention describes a method for the identification of a target bacteria from a sample isolated from a complex matrix such as food and enriched for the presence of the target. The method employs a method of picking colonies cultured from the sample such that the DNA from the cells may be immediately subjected to primer directed amplification after picking and without further processing. Amplification products are detected on the basis of detection of an intercalated fluorescent dye. Fluorescent signal generated from the amplification products is easily differentiated over background.

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TITLE

IDENTIFICATION OF TARGET BACTERIA BY FLUORESCENCE DETECTION OF PRIMER DIRECTED AMPLIFICATION PRODUCTS

FIELD OF THE INVENTION

This invention relates to the field of microbiology and molecular biology and particularly to methods for the detection of bacteria at the serotype level from complex matrices such as food.

BACKGROUND OF THE INVENTION

Food borne bacterial contamination is a prominent health hazard in developed as well as developing countries. For example, two to three million cases of *Salmonella* food poisoning are estimated to occur each year in the United States. Acute symptoms of this disorder include nausea, vomiting, diarrhea, cold chills, fever, and exhaustion. About 2,000-3,000 (0.1%) people die each year in the United States from *Salmonella* poisoning, these victims usually including infants, the sick, and elderly.

Poultry, red meat, seafood, eggs and any foods which contain these products particularly have the potential to carry bacterial pathogens. The challenge to a food microbiologist is to be able to recover and identify small numbers of these select pathogens from food products which often contain large numbers of a variety of other bacteria.

The traditional techniques for the detection and identification of microbes are based on the phenomenological or phenotype characteristics of the organism, such as its ability to metabolize certain specific nutrients, or its ability to bind with organism-specific antibodies. Such methods are intrinsically limited in their capabilities since (a) different organisms will require different growth and identification techniques; (b) the number of methods needed for detailed characterization of a number of different organisms will necessarily be unmanageably large; and, (c) the growth history and environment from which an organism is isolated can affect its phenotypic behavior.

Most of the microbiological testing today is performed using conventional culture techniques according to Bacteriological Analytical Manual (BAM) procedures. These are manual methods involving many steps and taking up to 14 days to obtain definitive results for particular organisms.

Culture-based testing begins with a food sample which is homogenized in a non-specific growth medium and allowed to incubate for 24 hours. This non-specific medium encourages growth of all microorganisms present in the sample and the recovery of injured organisms. The next step, selective growth, involves transfer of samples of non-selective broth into selective media tailored

for specific target organisms. The samples are incubated for an additional 24-48 hours. The selective media contain agents which suppress or even eliminate the growth of "background" organisms, while supporting the growth of the target pathogens. In the final growth step, several samples of the selective media are then streaked onto several solid, selective and differential media (agar). These agar media further suppress the growth of background organisms while supporting the growth of the target pathogen into isolated, differentiated colonies.

After a specified period of incubation, the plates are usually read for evidence of growth of target bacteria. The analysis of plated colonies is generally very time consuming, and often requires guesswork. If the plates contain clearly defined colonies having the appropriate morphological characteristics a battery of biochemical and/or serological tests must be performed for confirmatory identification and characterization of the suspects. If the colonies on the specific growth plate are not clearly defined, a re-streaking step may be necessary. If no colonies are observed after a specified incubation period, the food sample is considered to be negative for the specific pathogen.

Problems with the culture approach requiring the analysis of plated colonies are legion and include: elementary levels of information, inconsistent results, proliferation of methods and reagents unique to the target organism, long turn-around time, labor intensive protocols and operator technique dependency.

Non-growth, biotechnology-based technologies offer a potential solution to the time intensive colony analysis in growth based methods. These non-growth based technologies, referred to as rapid methods, continue to be refined in order to provide rapid test results with greater assurance of quality products, translating into higher throughput and cost savings for both the food manufacturer and the testing lab.

Two alternate test methods, immunoassay and nucleic acid probes, are increasingly used. Other emerging technologies are adenosine triphosphate (ATP) bioluminescence, conductance and impedance methods, flow cytometry, biosensors and DNA amplification.

The most common immunological methods involve some form of an enzyme reporter linked to an antibody for detection of a pathogen-specific antigen. In an immuno-reaction, the antibodies will bind to the antigen on the target microorganism on or in the reaction medium thus forming an antibody/antigen complex. The antigen is then detected by addition of a "conjugate", a second enzyme-labeled antibody that binds to the antibody/antigen complex to form a "sandwich". After a separation or washing step, the enzyme concentration is measured by adding a substrate solution and measuring the

resulting signal either visually or spectrophotometrically. A reading above a specified threshold indicates a positive result. Enzyme-Linked Immunosorbent assays (ELISAs) and Enzyme-Linked Fluorescent assays (ELFAs) are typical.

Nucleic acid (e.g., DNA or RNA) probes are also useful for detection of specific pathogens such as *Salmonella*, *Listeria* and *E. coli*. These assays use a calorimetric format to detect ribosomal RNA (thousands of copies of RNA are in each cell as opposed to a single copy of DNA). Therefore, the use of RNA as a target increases assay sensitivity. As with immunoassays, an enrichment step is still required. RNA probes that are specific for an organism are reacted with enriched samples that have been treated with a lysing reagent to release the target RNA from their cells. Two different probes (a detector probe and a capture probe) which hybridize to adjacent regions of the target RNA are used. The detector probe has a fluorescent label (fluorocein), while the capture probe has a "tail" of thymine nucleotides ("poly dT"). The two probes are allowed to hybridize with the target RNA. The entire hybridization complex is then captured onto a plastic dipstick that is coated with poly dA molecules (which are complementary to the poly dT tail on the capture probe). After a washing step, an enzyme-labeled anti-fluorocein antibody is added. Addition of a substrate produces a color change that can be detected in a spectrophotometer. The entire assay process is similar in many respects to an ELISA but involves additional steps.

Probe assays are more complex and expensive than immunoassays, requiring many more protocol steps as well as a fully equipped laboratory. Both types of assays are time intensive, complex and require a battery of reagents for implementation. A simpler, more rapid method is needed.

In contrast to immunological and probe technology, genetic methods of identification involving the amplification of serotype-specific DNA or RNA provide the promise of a standardized method, where the same basic approach can be applied universally to the identification of any microorganism. Thus, the presence or absence of any specific target microorganism can, in principle, be readily established at genus, species or subspecies level by establishing the presence or absence of appropriate genetic markers.

Methods of identifying food borne bacteria based on amplification of specific portions of a bacterial genome by polymerase chain reaction (PCR) are known in the art. For example Maher et al. (*Mol. Cell. Probes* (1995), 9(4), 265-76) teach a method for the identification of pathogenic bacteria such as *Listeria* by fluorescently detecting PCR products generated with species-specific primer sets. Similarly Yue et al. (U.S. 5,321,130), Higuchi R. G. (CA 2067909) and Sutherland et al. (U.S. 5,563,037) all disclose bacterial detection methods

using the incorporation of cyanine dyes into PCR amplification products for the detection of serotype specific products. Bassler et al. (*Appl. Environ. Microbiol.* (1995), 61(10), 3724-8) teach a *Listeria* detection method which relies on the hydrolysis of an internal flouorgenic probe to monitor the amplification of the target. Methods such as these are highly specific and sensitive but require a clean sample of target DNA. Application of these methods to analysis of food samples would require many of the cumbersome growth methods described above to produce a sample free of contaminating non-specific DNA (which will result in high background fluorescence) and other materials that will inhibit the PCR reaction.

In spite of the difficulties associated with fluorescence-based PCR amplification of target DNA from bacteria isolated from food, several methods have been developed. For example Cano et al. (*J. Appl. Bacteriol.* (1993), 75(3), 247-53) utilize a *L. monocytogenes* and *Salmonella* specific primer pair to amplify target DNA from milk samples wherein the amplification products were labeled with ethidium bromide. Identification was made on the basis of hybridization to an immobilized fluorescent labeled probe. Cano et al. (*J. Food Prot.* (1995), 58(6), 614-20) also teach a microwell assay for the detection of *Listeria* from food relying exclusively on amplification of a *Listeria* specific target DNA. The methods of Cano et al. do not teach selection of target bacteria from enriched colonies, and require extensive DNA isolation and purification prior to amplification.

Although the PCR based methods of Cano et al., are capable of detecting food borne pathogens from food matrices, they still require either a hybridization step or extensive purification and isolation of genomic DNA subsequent to amplification of the target in order to detect the fluorescently labeled amplification products. Such steps add to the complexity of the assay and in some cases require the prior generation and immobilization of suitable fluorescently labeled hybridization probes.

The problem to be overcome therefore is to develop a method for the identification of pathogenic bacteria that is free of the encumbrances of the growth-based and immunologically-based identification methods for colony analysis and maximizes the benefits of primer directed amplification techniques. Applicants have solved the stated problem by developing a method wherein colonies of target and non-target bacteria isolated from food samples may be picked and directly submitted to primer directed amplification techniques for the generation and fluorescence detection of target specific amplification products.

SUMMARY OF THE INVENTION

The present invention provides a method for identifying a target bacterium in a sample analysate, the analysate suspected of containing the target bacterium and having been cultured to produce at least one colony, the method comprising the steps of: (i) picking at least a portion of a bacterial colony by means of an individual capillary tube; (ii) dispersing the cells comprising the colony of step (i) into a suitable dispersing buffer; (iii) lysing the dispersed cells of step (ii) by contacting the dispersed cells with an effective amount of lysis buffer such that the DNA contained within the dispersed cells is released; (iv) producing an amplification reaction mixture by contacting the DNA released in step (iii) with a nucleic acid amplification composition comprising amplification primers useful for amplification of target DNA; (v) performing primer directed amplification on the released DNA of step (iii) to produce an amplification product mixture; and (vi) analyzing the amplification product mixture for the presence of target amplification products wherein the target amplification products contain an intercalating agent, the intercalating agent added at any time before step (vi).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a histogram of the results of analysis of *Salmonella* and background bacteria in fluorescence PCR assays. The assays were performed in the presence of the intercalating dye YO-PRO-1™ in order to determine the threshold fluorescence of labeled *Salmonella*-specific amplification products.

DETAILED DESCRIPTION OF THE INVENTION

The following terms may be used for interpretation of the claims and specification.

The term "target nucleic acid" or "target DNA" refers to a nucleic acid fragment that is detected by the present detection method and is indicative of the presence of a target bacterium. The target DNA is typically a unique portion of the target bacteria genome and specifically distinguishes the target bacteria from all other bacteria.

The term "amplification primer" or simply "primer" refers to a nucleic acid fragment or sequence that is complementary to at least one section along a strand of the target nucleic acid, wherein the purpose of the primer is to sponsor and direct nucleic acid replication of a portion of the target nucleic acid along that strand. Primers can be designed to be complementary to specific segments of a targeted sequence. In PCR, for example, each primer is used in combination with another primer forming a "primer set" or "primer pair"; this pair flanks the targeted sequence to be amplified. The term "primer", as such, is used generally

by Applicants to encompass any sequence-binding oligonucleotide which functions to initiate the nucleic acid replication process.

The term "amplification product" refers to specific DNA fragments generated from any primer-directed amplification reaction. The term "target amplification product" or "amplified target" refers to double stranded DNA ("dsDNA") that is generated from a specific target nucleic acid to be identified and quantified by means of primer directed amplification. Target amplification products may be produced by any primer directed amplification method. Further, it is understood that all methods of preparing suitable target amplification products are sufficiently controlled so as to eliminate spurious non-target amplification artifacts such as primer-dimer fragments, triple helices and the like. Target amplification products will generally be dsDNA and will be amenable to being bound by intercalating agents.

The term "primer-directed amplification" refers to any of a number of methods known in the art that result in amplification of nucleic acid molecules using the recognition of a specific nucleic acid sequence or sequences to initiate an amplification process. Applicants contemplate that amplification may be accomplished by any of several schemes known in this art, including but not limited to the polymerase chain reaction (PCR) or ligase chain reaction (LCR) and strand displacement amplification.

The term "nucleic acid amplification composition" refers to a composition comprising the ingredients necessary for performing nucleic acid amplification. Nucleic acid amplification compositions may be provided in a variety of forms including liquid mixtures as well as tableted reagents. If PCR methodology is selected, the amplification composition would include, for example, nucleotide triphosphates, two primers with appropriate sequences, DNA polymerase, suitable buffers and proteins.

The term "target bacteria" refers to the bacteria from which the target DNA is amplified. Target bacteria may be members of defined mixed cultures, or exist as contaminants in complex matrices. Target bacteria of particular interest are food-borne pathogens.

The term "non-target bacteria" will be used interchangeably with the term "background bacteria" and will refer to any bacteria that are found in the presence of the target bacteria but are not the target bacteria. Non-target bacteria may or may not be related genetically or biochemically to the target bacteria. Those non-target bacteria of most interest in the context of the present application are non-pathogenic food-borne bacteria.

The term "picking" refers to the process of removing a specified portion of a bacterial colony. As disclosed herein, the instant method provides a means whereby a specific and uniform number of bacterial cells is picked, thus leading to reproducibility of the instant method while minimizing spurious, non-specific results.

The term "dispersing buffer" means a buffer that maintains the cells intact and is useful for the dispersion of individual cells from a bacterial colony. Isotonic buffers such as buffered saline are particularly suitable.

The term "lysis buffer" as used herein refers to any aqueous solution suitable for use in the lysing of bacterial cells and releasing the cellular DNA.

The term "amplification reaction mixture" refers to the mixture of all the reagents and cellular material necessary to perform primer directed amplification of target DNA. Typically the amplification reaction mixture results from the mixing of the lysis buffer containing cellular DNA and the nucleic acid amplification composition.

The term "amplification product mixture" refers to the mixture formed after primer directed amplification has been performed on the amplification reaction mixture. If target DNA is present in the amplification reaction mixture, then the amplification product mixture will contain amplification products.

The term "intercalating agent" means a temperature-sensitive fluorescent agent capable of intercalating into nucleic acid molecules. The term "intercalating agent" will be used interchangeably with the term "dye". Intercalating agents emit a fluorescent signal when intercalated into the nucleic acid and will not generate any signal when not intercalated. Typical of intercalating agents are the unsymetrical cyanine dyes such as YO-PRO-1™, available from Molecular Probes, Inc. (Eugene, OR, USA).

The term "threshold fluorescence" refers to the level of fluorescence above which the presence of dye-labeled target amplification products are confirmed. Threshold fluorescence is determined empirically by the analysis of a multiplicity of primer directed amplification reactions performed on both target and non-target bacteria.

The term "matrix" or "complex matrix" will refer to any organic or inorganic material that will support the growth of a variety of microorganisms. The matrices of the present invention will be complex in nature and will comprise of a variety of different organic growth-supporting substances. Typical matrices include components of food matter, biological tissues, organic waste products, and the like.

The term "pre-enrichment growth" or "pre-enrichment culture" refers to the growth of target and non-target bacteria isolated from a complex matrix in a medium designed to resuscitate both classes of bacteria which have been injured or compromised by the sampling process. "Pre-enrichment media" will refer to either a liquid or solid media designed to encourage the growth of both target and background bacteria. The pre-enrichment media of the present invention is buffered to allow for the variations in pH of a variety of different food matrices.

The term "selective growth" or "selective enrichment culture" refers to the growth of target and non-target bacteria isolated from a pre-enrichment culture in a selective media. The selective medium is designed to specifically encourage the growth of the target bacteria over the contaminating background or non-target bacteria. "Selective growth media" will refer to either a solid or liquid growth media specifically formulated to enhance the growth of the target bacteria and discourage the growth of background bacteria. Selective media capitalize on the specific nutrient requirements and the resistance to certain selective agents of the target bacteria to produce selective growth.

The term "sample analysate" refers to a sample of bacteria taken from a selective enrichment culture.

Utility Statement

The instant invention affords detection of target organisms, particularly bacteria, in the presence of contaminating food matrices and non-target bacteria. The method is particularly useful for the detection of pathogenic bacteria in the food preparation, agricultural and live stock industries, and also has application for medical and veterinary diagnostics.

The present invention defines a process for the detection of target bacteria from a complex matrix by means of (i) target enrichment, consisting of consecutive pre-enrichment and selective cultures followed by (ii) colony picking, (iii) cell lysis, and finally (iv) amplification of target DNA. Amplification products are detected by means of a signal generated by an intercalating agent bound to the target amplification products.

Target Enrichment

The minimum industry standard for the detection of food-borne bacterial pathogens is a method that will reliably detect the presence of one pathogen cell in 25 g of food matrix. In order to meet this stringent test, enrichment methods and media have been developed to enhance the growth of the target pathogen cell in order to facilitate its detection.

Typically at least two enrichment steps are preferred. The first step is a "pre-enrichment step" wherein the food sample is enriched in a nutritious,

nonselective broth medium to restore injured target cells to a stable condition and to promote growth. Next, a sample of the pre-enrichment broth is added to a selective enrichment broth, wherein the sample is further enriched in a growth-promoting medium containing selective reagents. Selective enrichment broth allows a continued increase of target bacteria while simultaneously restricting proliferation of most other background, non-target bacteria. Examples of selective media useful for the selective enrichment of *Salmonella* are tetrathionate broth and selenite cysteine broth. Media may be defined for virtually any known target bacteria, see for example Bacteriological Analytical Manual, 8th Edition, Association of Official Analytical Chemists, Arlington, VA (1995).

Although the above process for target enrichment is typical, it will be appreciated that it represents only one possible method and that other systems for the selective enrichment of a specified target bacteria will be dependent on the unique requirements of the specific target, all of which is well known to the skilled artisan in combination with standard protocols as given in the Bacteriological Analytical Manual *supra*.

Colony generation

Following target cell enrichment, samples of the selective cultures are plated on a solid medium for colony generation. Solid media are selected so as to further enrich the growth of target bacteria while inhibiting the growth of background or non-target species. A variety of media are known in the art. For example, media that are particularly suited for the selection of *Salmonella* over background bacteria include xylose lysine desoxycholate (XLD), bismuth sulfite (BS), and Hektoen enteric (HE) agars.

Methods of bacterial plating are common and well known in the art and a variety of suitable solid media are commercially available and in the public domain (see for example Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989); Bacteriological Analytical Manual, 6th Edition, Association of Official Analytical Chemists, Arlington, VA (1984)).

Colony Picking

Colonies grown on solid media are picked, lysed and submitted to a protocol for primer directed amplification of target DNA. The method of picking the colonies is an essential element of the present invention.

The present method allows for the optimum number of cells to be submitted to the primer directed amplification protocol. Higher numbers of cells result in excessive fluorescent background and the introduction of materials

known to inhibit DNA amplification. Lower numbers of cells result in insufficient amplification of target DNA.

Colonies were picked using a 5 uL glass capillary tube. Capillary tubes having an outside diameter ("O.D.") of between 0.026 in. and 0.0965 in are preferred in the present invention where an O.D. of about 0.0375 in. is most preferred. The particular capillary chosen for the experiments described herein was a 5 uL Microcap capillary (Drummond Scientific Co., Broomall, PA) of about 1.26 inches in length. Picking colonies with this device delivered between $1x10^6$ and $2x10^7$ cells to the lysis buffer.

Other design aspects of these capillaries lend utility. The small size, both in length and diameter allow placement of at least 12 capillaries in a single tube for resuspension of the collected cells by vortexing. This allows for rapid processing of multiple colonies and greater reproducibility in resuspending cells as opposed to individual sample, manual methods. Further, this picking process eliminates the need for quantification methods such as optical density measurements, commonly used in biochemical assays to deliver the required number of cells. Also, the diameter of the capillary acts as a gauge which is used to determine whether a colony is large enough to yield the required number of cells. Finally, the inert nature of glass guards against release of PCR inhibitory chemicals from the picking device.

Primer Directed Amplification Of Target DNA

Target DNA may be from any source where it is of value to discern the presence of a double-stranded nucleic acid fragment that is unique to a particular organism or is defining for a specific genetic trait. In one embodiment of the instant invention, target DNA is amplified to produce target amplification products from pathogenic bacterial organisms known to contaminate food. Of particular interest will be DNA isolated from organisms such as members of the following genera: *Listeria*, *Salmonella*, *Clostridium* and *Escherichia*.

Applicants contemplate that amplification may be accomplished by any of several schemes known in this art, including but not limited to the polymerase chain reaction (PCR), ligase chain reaction (LCR) or strand displacement amplification (SDA). If PCR methodology is selected, the amplification composition would include for example, nucleotide triphosphates, two primers with appropriate sequences, suitable buffers, DNA polymerase and proteins. These reagents and details describing procedures for their use in amplifying nucleic acids are provided in U.S. Patent No. 4,683,202 and U.S. Patent No. 4,683,195. If LCR methodology is selected, then the nucleic acid amplification compositions would comprise, for example, a thermostable ligase,

e.g., *T. aquaticus* ligase, two sets of adjacent oligonucleotides wherein one member of each set is complementary to each of the target strands. Tris-HCl buffer, KCl, EDTA, NAD, dithiothreitol and salmon sperm DNA. (See, for example, Tabor, S. and Richardson. C. C. (1985) *Proc. Acad. Sci. USA 82*, 1074-1078). If the SDA methodology is employed, amplification may be accomplished using either one or two short primers containing a site for *HincII* digestion, an exonuclease deficient DNA polymerase, *HincII* restriction enzyme and the bases dGTP, dCTP, dTTP and deoxyadenosine 5'[a-thio]triphosphate (dATP[aS]. The SDA protocol including the necessary materials is outlined in Walker, et al., *Proc. Natl. Acad. Sci. U.S.A.*, 89, 392, (1992).

It should be noted that all methods of preparing suitable target amplification products are sufficiently controlled so as to eliminate spurious non-target amplification artifacts such as primer-dimer fragments, triple helices and the like.

Reagents necessary for target amplification may be supplied to the present method in either solution or in tableted form. For example, suitable tableted reagents for the PCR amplification of *Salmonella* specific target DNA are described in International Patent Application No. PCT US96/15085. Where fluorescence detection is chosen as a means for the detection of amplification products, it may bė useful to incorporate "positive control" and "negative control" tableted reagents in order to calibrate the amplification reaction and the monitor background fluorescence. For example a "sample tablet" will contain all the necessary reagents to amplify a specified target DNA, including appropriate primers and a suitable DNA polymerase. "Positive control" tablets will contain all the reagents encompassed within the sample tablet with the addition of a specified amount of target DNA. Addition of the positive control tablet into the PCR reaction will result in the amplification of the target DNA within the tablet thereby providing a reference for both the PCR reaction and for fluorescence detection. The "negative control" tablet contains all the reagents encompassed in the sample tablet with the exception of the DNA polymerase. The presence of the negative control tablet in the PCR reaction serves as a reference control for background fluorescence.

<u>Intercalating Agents</u>

The present method uses an intercalating agent capable of binding to dsDNA and emitting a fluorescent signal distinguishable from the signal generated when unbound or bound to single stranded DNA. A variety of suitable intercalating agents are known in the art such as propidium iodide (PI) and ethidium bromide (EB) (Sailer et al., *Cytometry* (1996), 25(2), 164-172), Oxazole Yellow (EP 714986), TOTO (1,1'-(4,4,7,7-tetramethyl-4,7-diazaundeca-

methylene)-bis-4-[3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene]-quinolinium tetraiodide), a homodimer of thiazole orange (Axton et al., *Mol. Cell. Probes* (1994), 8(3), 245-50) oxazole orange (YOYO; Srinivasan et al., *Appl. Theor. Electrophor.* (1993), 3(5), 235-9) as well as the cyanine dyes (U.S. Patent No. 5,563,037). Preferred in the present method are the unsymmetrical cyanine dyes such as are discussed in U.S. Patent No. 5,563,037, U.S. Patent No. 5,534,416, and U.S. Patent No. 5,321,130 hereby incorporated by reference.

Cyanine dyes are particularly suited for use in the present method since they possess binding constants for DNA low enough not to inhibit amplification by most primer directed amplification methods but high enough to still give a detectable signal. Preferred binding constants for the cyanine dyes useful in the present invention are from about from about $1x10^4$ to about $5x10^5$ (molar^{-1}).

Most suitable is the cyanine dye YO-PRO-1™ (Quinolinium, 4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(trimethylammonio)-propyl]-,diiodide) available from Molecular Probes, Inc. (Eugene, OR, USA). YO-PRO-1™ is particularly suited for use in the present invention due to its high extinction coefficient, near zero fluorescence when unbound, suitable binding affinity to double-stranded DNA and reasonable photostability. YO-PRO-1™ is sufficiently resistant to the elevated processing temperatures at the time intervals used to provide an effective signal in use.

The intercalating agent can be provided at any step of the method prior to fluorescence detection. For example, the intercalating agent may be present in the buffer into which cells comprising picked bacterial colonies are dispersed. In another embodiment, the intercalating agent can be present in the buffer containing the lysis reagent. In a further embodiment, the intercalating agent can be introduced into the method as a component of the amplification composition. Finally, the intercalating agent can be added to the amplification product mixture just prior to analysis by fluorescence detection.

The intercalating agent chosen for use in the instant method may be temperature sensitive; i.e., the binding affinity of the intercalating agent for dsDNA and hence the magnitude of the fluorescent signal emitted may vary with temperature. Accordingly, it is readily apparent to one skilled in the art that instrument calibration, positive and negative controls and samples must all be assayed under controlled temperature conditions. Alternatively, a mathematical algorithm may be developed in order to compensate for variations in ambient and calibration temperatures. For example, the following algorithm comprises a simple linear multiplier that calculates the fluorescence value at a standard calibration temperature (FIUc) as a function of the ambient temperature at which

sample measurements are taken (t) and the fluorescence intensity units (FIUt) recorded at that temperature:

FIUc = FIUt(TCF). wherein

FIUc = the calculated fluorescence value;

FIUt = the measured fluorescence value at a given ambient temperature (t); and

TCF = ((0.25 + 0.05(t))/1.45.

This algorithm produces a constant result over a temperature range of 15-35°C.

Target amplification

In order to identify a target by the present method. bacterial. cells picked according to the above mentioned methods are lysed in a lysis buffer to release cellular DNA. DNA is then amplified according to a standard method for primer directed amplification. Typically, PCR is used and follows a standard thermo-cycling procedure in the presence of an appropriate nucleic acid amplification composition. A suitable nucleic acid amplification composition will contain for example, dATP, dCTP, dGTP, dTTP, target specific primers and a suitable polymerase. Primers will be selected to specifically amplify target DNA. If nucleic acid composition is in liquid form, suitable buffers known in the art are used. (Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)). If the composition is contained in a tabletted reagent, then typical tabletting reagents are included as described in PCT US96/15085.

The intercalating agent YO-PRO-1™ can be included in the PCR reaction. YO-PRO-1™ is added to the sample to give a final dye concentration of about 3 uM. Thermocycling proceeds according to typical cycling times and temperatures.

Description of the preferred embodiments

The method of the invention begins with the collection of samples suspected of containing target bacteria and submission of those samples to target enrichment protocols. Samples are first cultured in a non-selective pre-enrichment medium and incubated for about 24 hours at about 37 °C. Following pre-enrichment, samples of the pre-enrichment culture are diluted into a selective medium to enrich the growth of the target bacteria. Aliquots of the selective culture are then streaked on appropriate solid media for colony generation. A defined number of bacteria are then picked by means of an individual 5 uL capillary tube from at least one colony on each plate. Capillary tubes from each pick are deposited in a container and immersed in an suitable dispersing buffer. Optionally the dispersing buffer may contain an intercalating agent capable of intercalating into DNA and emitting a fluorescent signal. The container is then

sealed and agitated to disperse the cells from the capillary tubes into the amplification composition.

Following agitation and dispersion of the cells, samples of the solution are contacted by a lysis buffer where the cells are lysed and cellular DNA is released. The lysis buffer may optionally contain an effective concentration of intercalating agent. Lysis of the cells may occur by any method, including differential osmotic pressure, mechanical means such as sonication or heat.

Reagents necessary for the primer directed amplification (typically PCR) of target DNA may be added after cell lysis. Such reagents will typically include nucleotide triphosphates, two primers with appropriate sequences, suitable buffers, DNA polymerase and proteins. These reagents may be provided in either solution or tableted form. Preferred are the use of tableted PCR reagents. Three types of tablets are used: a sample tablet, a positive control tablet and a negative control tablet. Sample tablets contain all reagents necessary for the amplification of the target. Positive control tablets contain the same reagents as the sample tablet with the addition of a specified amount of target DNA. Negative control tablets contain the same reagents as the sample tablet with the exception of the DNA polymerase. All tablets are processed in the PCR reaction under identical conditions. The positive control tablet serves as a check on the efficacy of the reaction as well as a monitor of expected fluorescence levels. The negative control tablet is an indication of background fluorescence produced by non-productive PCR reagents.

Following addition of the PCR reagents, the amplification protocol is performed in a sealed reaction vessel. Sealing of the vessel is important to prevent carryover contamination. If target DNA is present, amplification products are produced and products are detected and quantified.

It will be appreciated that the present invention may also be practiced in kit form. A typical kit will provide all consumable/disposable elements required for multiple analyses. Components for a Kit may include for example, Microcap Tubes, Sample Tube Assemblies consisting of clear PCR tubes with PCR caps having sample PCR tableted reagents enclosed, Lysis Buffer, a Control Tablet Package consisting of a first set of capped PCR tubes having negative control PCR tableted reagents enclosed and a second set of capped PCR tubes having positive control PCR tableted reagents enclosed, a YO-PRO-1™ Package, and Calibration Tubes consisting of a first set of tubes filled with a 1:258 dilution of Estapor® fluorescent pink polystyrene particles(Stock Code D0001852CF; Bangs Laboratories, Inc., Carmel, IN), and a second set of tubes filled with lysis buffer.

The present invention is further defined in the following Example. It should be understood that this Example, while indicating a preferred embodiment of the invention, is given by way of illustration only. From the above discussion and this Example, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLE

GENERAL METHODS

Procedures for primer directed amplification are well known in the art. Techniques suitable for use in the following examples may be found in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989).

Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994) or Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA. or Bacteriological Analytical Manual. 8th Edition, Association of Official Analytical Chemists, Arlington, VA (1995). All reagents and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

A Perkin Elmer GeneAmp Cycler 9600 (Perkin-Elmer, Branchburg N.J.) was used for all PCR reactions.

The cyanine intercalating agent YO-PRO-1™ was obtained from Molecular Probes (Eugene, OR).

Salmonella-specific PCR reagents used in the following examples were obtained from Qualicon, L.L.C., Wilmington, DE (part no. 17410530) and are included in the *Salmonella* screening kit (catalog number 17720519).

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "uL" means microliters, "mL" means milliliters, "L" means liters, "FIU" means fluorescent intensity units.

Cell Strains

The following *Salmonella* strains were used in the examples provided herein: *S. enteritidis* (737), *S. typhimurium* (897), *S. virchow* (1247), *S. hadar* (1268), *S. kedougou* (1254), *S. heidelberg* (1239), *S. montevideo* (1260), and *S. infantis* (732). Non-target background organisms employed herein include *Citrobacter diversus* (2560), *Citrobacter spp.* (217), *E. coli* (5108), *E. coli* (5111), *Hafnia alvei* (4965), *Hafnia alvei* (2010), *Proteus mirabilis* (2321), and *Proteus vulgaris* (2323). All bacterial strains were obtained from the Qualicon L.L.C. culture collection. Numbers in parentheses indicate internal Qualicon L.L.C. culture designations.

Solid Selective Media

Three types of solid media were used for the generation of colonies from target (*Salmonella*) and non-target bacteria. They consisted of xylose lysine desoxycholate (XLD), bismuth sulfite (BS), and Hektoen enteric (HE) agars. Protocols for their preparation may be found in Bacteriological Analytical Manual, 8th Edition, Association of Official Analytical Chemists, Arlington, VA (1995).

Buffers and Amplification Reagents

Lysis buffer used in this example consisted of 50 mM Tris, 28 mM KCl, 3 mM $MgCl_2$, 0.1% Triton X-100, pH 8.3 containing 3 uM YO-PRO-1™.

PCR reagent components consisted of:

36 nM of each of the following amplification primers:

SEQ ID NO:1	5'-TAG CCG GGA CGC TTA ATG CGG TTA AC-3'
SEQ ID NO:2	5'-CTT TAC CGC TTC CAG TGT GGC CTG AA-3'

3 mM of magnesium,

160 uM of dNTP, and

1.5 units of Taq™ polymerase per assay.

The PCR buffer was comprises 50 mM Tris, 28 mM KCl, 0.1% Tween-20 buffer, pH 8.3.

Fluorometer Calibration

A fluorometer, equipped with a tungsten-halogen lamp, a 485 nm excitation filter and a 535 nm emission filter was used for detection of fluorescence emitted from amplification product.

Reagents used for the calibration of the fluorometer are as follows:

(i) DNA standard: 500 bp double strand DNA fragment (BioVentures, Inc.; Catalogue No. M500).

(ii) lysis/dye buffer: lysis buffer containing 3 uM YO-PRO-1™.

The DNA standard was prepared at three different concentrations: 0 ng DNA, 200 ng DNA/50 uL and 400 ng DNA/50 uL. A standard curve was generated by measuring the fluorescence emitted from the three concentrations of DNA standards. The mean value of all three readings for each concentration was calculated and the results are tabulated below in Table I:

TABLE I
Calibration Results

Calibrator	[DNA] (ng/assay)	Signal (FIU)	Signal (FIU)	Signal (FIU)	Mean signal (FIU)
Level 1	0	0.025	0.031	0.024	0.027
Level 2	200	3.304	3.185	3.14	3.21
Level 3	400	6.244	5.764	5.708	5.905

Determination Of Threshold Fluorescence

In order to determine threshold values of fluorescence to distinguish YO-PRO-1™ labeled amplification products, a multiple parameter combination study was conducted. Data from 620 *Salmonella* PCR assays in the presence of the intercalating dye YO-PRO-1™ were analyzed using both analysis of variance and histogram techniques (JMP: Statistical Discover Software, SAS Institute, Inc. Cary, NC) to determine the fluorescence threshold value for *Salmonella*. These analyses were done for each strain (*Salmonella* and non-*Salmonella*) and provided summaries of variance components. The results of analysis can be seen in the attached histogram, Figure 1. For *Salmonella*, a threshold value of 1.5 FIU was determined.

EXAMPLE

Identification Of *Salmonella* From Mixed Cultures

The ability of the instant method to specifically identify each of a panel of eight different target *Salmonella* strains present in a mixed culture with non-target, non-*Salmonella* strains was determined.

One colony of each of the *Salmonella* strains was picked per test. A total of 11 colonies representing the non-*Salmonella* strains were picked and added to each of the tubes containing the individual *Salmonella* colonies. One control *Salmonella* strain was tested to monitor consistency of the method. The fluorescence (FIU) data were recorded and measured against a pre-determined threshold value for *Salmonella*. All assays were performed in triplicate.

Assay for *Salmonella* by Fluorescence PCR Detection

A. The following Reagents and Accessories were employed:

Salomella strains cultured on XLD plates

Non-*Salmonella* strains cultured on HE, XLD, and BS plates

Capillary tubes (5 uL size)
Cell suspension tubes (1.5 mL, Eppendorf)
Cell lysis tubes (2 mL capacity with screwcap)
PCR vials (std. size - 0.2 mL)
PCR sample tablets
PCR positive control tablets
PCR negative control tablets
YO-PRO-1™ reagent (5.26 mL/tube)
Lysis buffer

B. Equipment used in the instant example included:

1) Perkin Elmer GeneAmp™ 9600 thermal cycler
2) Heating water bath (up to 95 °C)
3) Fluorometer

C. Lysis/Dye solution was prepared as follows. A 3 uM YO-PRO-1™ solution was prepared by adding 1.75 mL of lysis buffer into the YO-PRO-1™ tube (as received from the manufacturer). PCR reagents tubes were prepared by pipetting 1 mL lysis buffer and 200 uL of the 3 uM YO-PRO-1™ solution into tubes containing either a sample PCR tablet (experimental), a positive control tablet or a negative control tablet.

D. Cell picking procedure.

The following *Salmonella* strains were separately cultured on XLD plates: *S. enteritidis* (737), *S. typhimurium* (897), *S. virchow* (1247), *S. hadar* (1268), *S. kedougou* (1254), *S. heidelberg* (1239), *S. montevideo* (1260), and *S. infantis* (732).

Each of following background bacterial strains were separately cultured on XLD, HE and BS plates: *Citrobacter diversus* (2560), *Citrobacter spp* (217), *E. coli* (5108), *E. coli* (5111), *Hafnia alvei* (4965), *Hafnia alvei* (2010), *Proteus mirabilis* (2321), and *Proteus vulgaris* (2323).

Colonies were picked from the culture plates as follows:

1) The capillary container was held upside down and a capillary was removed from the hole by grasping one end, making sure not to touch the other end of the capillary.

2) One isolated colony was touched with the free end of the capillary by holding the capillary perpendicular to the plate and gently pressing in the center of the colony.

3) The capillary was removed from the colony and dropped into the cell suspension tube.

Evaluation of ability of the instant method to detect target bacteria in the presence of non-target bacteria was performed by picking one target (*Salmonella*) bacterial colony and eleven non-target bacterial colonies to the same tube. Colonies of non-target bacteria were picked at random. The tube was then capped and vortexed at full speed for at least 5 seconds. This method of picking was repeated for each *Salmonella* strain tested. Assay response to each of the non-target bacterial strains was measured by picking twelve colonies of the same non-target strain to a single assay tube. Colonies of non-target bacteria were picked so that four colonies growing on each of the three growth media were picked to the same tube. This picking protocol was repeated for all of the non-target bacterial strains tested.

E. <u>Cell Lysis</u>:

Cell lysis was performed as follows:

1) 10 uL of each colony suspension was removed from each sample tube and placed in a lysis tube containing 190 uL of lysis/dye solution.

2) Each tube was tightly capped and vortexed to mix the contents.

3) Tubes were incubated for 10 min in a water bath at 95°C.

4) Following the incubation period, samples were allowed to cool to room temperature.

F. <u>PCR Amplification</u>

PCR amplification was performed as follows:

1) The thermal cycler was programmed as follows:

HOLD:	94 °C for 2 min., one cycle
CYCLE:	94 °C for 15 seconds,
	72 °C for 2 min.,
	35 cycles
HOLD:	72 °C for 7 min., one cycle
HOLD:	25 °C forever.
Sample vol.:	50 uL

3) 50 uL of each lysed sample was pipetted into each PCR sample tube.

4) 50 uL of each sample was pipetted into positive and 50 uL into negative control PCR tubes.

5) All tubes were capped and placed into the thermal cycler, and the program was initiated.

G. <u>Fluorescence Detection</u>

The fluorometer was permitted to warm up and was calibrated. PCR reaction samples were removed from the thermal cycler and allowed to cool to room temperature. Samples were read at an excitation of 485 nm and an emission

of 535 nm. Readings were compared against a previously determined threshold value.

H. Interpretation of Data

The PCR Fluorescence threshold value for *Salmonella* was previously determined to be 1.5 FIU. As an internal control. the positive control PCR fluorescence reading was maintained above threshold and the negative control PCR was maintained below the threshold.

Any sample PCR fluorescence reading that equaled or exceeded the threshold value was read as positive, indicating the presence of an amplification product. Where the reading was less than the threshold value, the result was negative, indicating no amplification product generated. Results are tabulated in Table II.

TABLE II

Sample No.	Bacterial species	PCR product signal (FIU)
1	*S. entertidis* 737	7.1
2	*S. typhimurium* 897	6.7
3	*S. vichow* 1247	6.3
4	*S. hadar* 1268	7.2
5	*S. kedogou* 1254	5.7
6	*S. heidelberg* 1239	6.2
7	*S. Montevideo* 1260	6.9
8	*S. infantis* 732	6.3
9	*Citrobacter diversus* 2560	0.65
10	*Citrobacter sp.* 217	0.5
11	*E. coli* 5111	0.59
12	*E. coli* 5108	0.62
13	*Hafnia alvei* 4965	0.79
14	*Hafnia alvei* 2010	0.72
15	*Proteus mirabillis* 2321	0.59
16	*Proteus vulgaris* 2323	0.56

As can be seen from the data presented above. the PCR product signal in all eight *Salmonella* species (Sample Nos. 1-8) was above the established threshold (1.5 FIU). The PCR product fluorescence signal in all non-*Salmonella* species (Sample Nos. 9-16) was well below the threshold. Thus the present method was effective in confirming the presence target colonies, even in the context of at several fold greater numbers of non-target bacteria.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: TSENG, SUSAN YEN-TEE
BARBOUR, WILLIAM MARK

(ii) TITLE OF INVENTION: IDENTIFICATION OF TARGET BACTERIA BY FLUORESCENCE DETECTION OF PRIMER DIRECTED AMPLIFICATION PRODUCTS

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 1007 MARKET STREET
(C) CITY: WILMINGTON
(D) STATE: DELAWARE
(E) COUNTRY: UNITED STATES OF AMERICA
(F) ZIP: 19898

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: DISKETTE, 3.50 INCH
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: MICROSOFT WINDOWS 95
(D) SOFTWARE: MICROSFOT WORD FOR WINDOWS 95

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/650074
(B) FILING DATE: MAY 17, 1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: MAJARIAN, WILLIAM R.
(B) REGISTRATION NUMBER: 41,173
(C) REFERENCE/DOCKET NUMBER: MD-1064-A

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 302-992-4926
(B) TELEFAX: 302-773-0164

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TAGCCGGGAC GCTTAATGCG GTTAAC 26

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTTTACCGCT TCCAGTGTGG CCTGAA 26

What is claimed is:

1. A method for identifying a target bacterium in a sample analysate, the analysate suspected of containing the target bacterium and having been cultured to produce at least one colony, the method comprising the steps of:

(i) picking at least a portion of a bacterial colony by means of an individual capillary tube;

(ii) dispersing the cells comprising the colony of step (i) into a suitable dispersing buffer;

(iii) lysing the dispersed cells of step (ii) by contacting the dispersed cells with an effective amount of lysis buffer such that the DNA contained within the dispersed cells is released;

(iv) producing an amplification reaction mixture by contacting the DNA released in step (iii) with a nucleic acid amplification composition comprising amplification primers useful for amplification of target DNA;

(v) performing primer directed amplification on the released DNA of step (iii) to produce an amplification product mixture; and

(vi) analyzing the amplification product mixture for the presence of target amplification products

wherein the target amplification products contain an intercalating agent, the intercalating agent added at any time before step (vi).

2. The method of Claim 1 wherein the capillary tube a five microliter tube.

3. The method of Claim 2 wherein the capillary tube has an O.D. from about 0.026 inches to about 0.0965 inches.

4. The method of Claim 3 wherein the capillary tube has an O.D. of about 0.0375 inches.

5. The method of Claim 1 wherein the picking results in removal of from about $1x10^6$ to about $2x10^7$ cells from the colony.

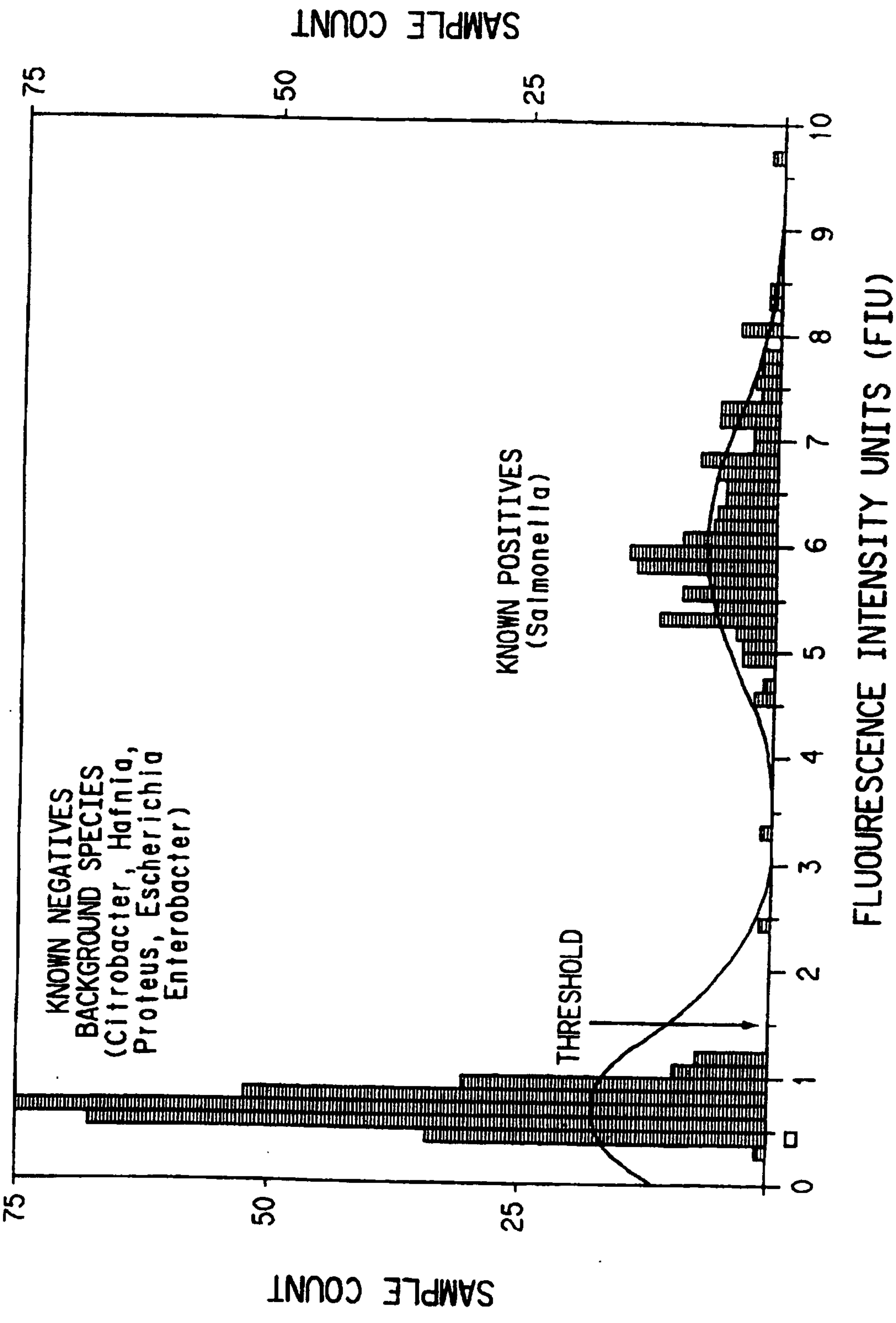
6. The method of Claim 1 wherein the primer directed amplification is a member selected from the group consisting of polymerase chain reaction, ligase chain reaction and strand displacement amplification.

7. The method of Claim 1 wherein the amplification primers amplify a unique portion of the genome a bacterial genus, the genus selected from the group consisting of *Salmonella*, *Listeria*, *Eschericia*, and *Clostridium*.

8. The method of Claim 1 wherein the intercalating agent is an unsymmetrical cyanine dye.

9. The method of Claim 8 wherein the unsymmetrical cyanine dye is YO-PRO-1™.

FIG.1


SAMPLE COUNT
75
50
25
KNOWN NEGATIVES
BACKGROUND SPECIES
(Citrobacter, Hafnia,
Proteus, Escherichia
Enterobacter)
THRESHOLD
KNOWN POSITIVES
(Salmonella)
0
1
2
3
4
5
6
7
8
9
10
FLUOURESCENCE INTENSITY UNITS (FIU)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/07958

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 684 316 A (JOHNSON & JOHNSON CLINICAL DIA) 29 November 1995 cited in the application see the whole document	1-10
X	GB 2 282 138 A (TOSOH CORP) 29 March 1995 see the whole document	1-8
X	EP 0 512 334 A (HOFFMANN LA ROCHE) 11 November 1992 cited in the application see the whole document	1-8
P,X	WO 97 11197 A (DU PONT) 27 March 1997 see abstract and claims (esp. claim 13)	1-10

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
1 September 1997	11.09.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Müller, F

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/07958

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0684316 A	29-11-95	CA 2148140 A	30-10-95
		CN 1114359 A	03-01-96
		JP 8089299 A	09-04-96
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GB 2282138 A	29-03-95	FR 2710920 A	14-04-95
EP 0512334 A	11-11-92	AU 665185 B	21-12-95
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